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Synergistic effect of pro-inflammatory TNFα and IL-17 in periostin mediated collagen deposition: Potential role in liver fibrosis

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ABSTRACT

Background: The pro-inflammatory cytokines, tumor necrosis factor (TNF)-α, and interleukin (IL)-17, have been implicated in the pathogenesis of liver fibrosis. In this study, we investigated the role of TNFα and IL-17 toward induction of profibrotic factor, periostin.
Methods: HepG2 cells were cultured and treated with inflammatory cytokines, TNFα and IL-17. Computational promoter sequence analysis of the *periostin* promoter was performed to define the putative binding sites for transcription factors. Transcription factors were analyzed by Western blot and Chromatin Immunoprecipitation. Periostin and transcription factor expression analysis was performed by RT-PCR, Western blot, and fluorescence microscopy. Type I collagen expression from fibroblast cultures was analyzed by Western blot and Sircol soluble collagen assay.
Results: Activation of HepG2 Cells with TNFα and IL-17 enhanced the expression of periostin (3.5 and 4.4 fold, respectively $p < 0.05$) compared to untreated cells. However, combined treatment with both TNFα and IL-17 at similar concentration demonstrated a 13.3 fold increase in periostin ($p < 0.01$), thus suggesting a synergistic role of these cytokines. *Periostin* promoter analysis and specific siRNA knock-down revealed that TNFα induces periostin through cJun, while IL-17 induced periostin via STAT-3 signaling mechanisms. Treatment of the supernatant from the cytokine activated HepG2 cells on fibroblast cultures induced enhanced expression of type I collagen (>9.1 fold, $p < 0.01$), indicative of a direct fibrogenic effect of TNFα and IL-17.
Conclusion: TNFα and IL-17 induced fibrogenesis through cJun and STAT-3 mediated expression of profibrotic biomarker, periostin. Therefore, periostin might serve as a novel biomarker in early diagnosis of liver fibrosis.

1. Introduction

Chronic liver disease is one of the top fifteen leading causes of death in the United States (Murphy et al., 2010). Most common causes of chronic liver disease include: viral hepatitis, alcoholism, metabolic and autoimmune diseases (Schuppan and Afdhal, 2008). The progression from healthy liver tissue to cirrhosis is mediated by a chronic inflammatory reaction eventually leading to the excess deposition of extracellular matrix proteins (Friedman, 2008). The inflammatory reaction is considered to be the key predictor of disease progression (Argo et al., 2009; Asselah et al., 2005). The accumulation of ECM proteins distorts the hepatic architecture by forming a fibrous scar, and the subsequent development of nodules of regenerating hepatocytes leading to cirrhosis. Hepatic fibrosis is generally considered to be an irreversible process manifested by a collapse in the hepatic parenchyma and its substitution with a collagen-rich tissue (Bataller and Brenner, 2005). Animal models and cell culture studies on liver fibrogenesis led to the

Abbreviations: TNF, tumor necrosis factor; IL, interleukin; HepG2, liver hepatocellular carcinoma cell line; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; NaCl, sodium chloride; NaF, sodium fluoride; ChIP, chromatin immunoprecipitation; qRT-PCR, quantitative real time polymerase chain reaction; cJun, c-Jun N-terminal kinases; MMP, matrix metaloproteinase.

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identification of key fibrotic biomarkers (Hayashi and Sakai, 2011). Research and understanding in the pathogenesis of liver fibrosis could aid the development of novel therapeutic strategies to prevent and possibly reverse the disease progression.

Inflammation is an important starting event of liver fibrosis (Czaja, 2014). Inflammatory cytokine levels are elevated independently of the etiology of the underlying liver disease (Tilg et al., 2006). Among the pro-inflammatory cytokines, tumor necrosis factor-alpha (TNFα) is a potent cytokine that exerts pleiotropic inflammatory and immunological functions by triggering downstream signaling events leading to hepatic fibrosis (Osawa et al., 2013). Levels of circulating TNFα are increased in patients with liver fibrosis and are associated with poor prognosis (Odeh et al., 2005). Further in an experimental cholestasis induced by bile duct ligation TNFα and IL-6 were elevated indicating the pathogenic nature of these cytokines (Gabele et al., 2009; Odeh, 2007). Hepatic macrophages in chronic liver inflammation exhibit high levels of TNFα expression (Zimmermann et al., 2012). TNFα has also been demonstrated to induce fibrosis in other models. In a model of pulmonary fibrosis, TNFα receptor knockout mice were protected from the development of fibroproliferative lesions (MacEwan, 2002). Also, hepatic injury following administration of the hepatotoxin carbon tetrachloride was inhibited in TNFα knockout mice (Sudo et al., 2005). Along with TNFα alpha another inflammatory cytokine IL-17 has been implicated in the liver fibrosis (Tang et al., 2011). IL-17A plays a critical role in neutrophil recruitment, angiogenesis, inflammation, and autoimmune disease that has been previously described extensively (Miyamoto et al., 2003), including in pulmonary and cardiac fibrosis via IL-17 receptor mediated signaling (Gasse et al., 2011; Zhu et al., 2011). Although these cytokines induce fibrotic events, to date, very limited data is available on fibrotic biomarkers with therapeutic value.

Periostin is a 90-kD secretory protein, playing an important role in the development of bones, teeth and tumor progression (Hamilton, 2008; Ruan et al., 2009). Recent evidence suggests that expression of periostin is involved in various pathophysiological statuses of fibrosis, including the healing process in myocardial infarction, pulmonary fibrosis and bone marrow fibrosis (Oka et al., 2007; Oku et al., 2008). Studies from lung models indicate that periostin contributes to the formation of fibrosis in response to inflammatory cytokines (Sidhu et al., 2010). In this report, we demonstrate that TNF and IL-17 exert a synergistic effect on HepG2 cells through two different signaling pathways leading to enhanced periostin expression and eventual induction of Type I collagen expression, a potential pathogenic mechanism in liver fibrosis.

2. Materials and methods

2.1. Cell culture

HepG2 cells were obtained from the American Type Culture Collection (HB-8065, ATCC, Manassas, VA) and cultured in cell basal essential media (30-2003, ATCC, Manassas, VA) along with the media supplements as recommended by the manufacturers (Sarma et al., 2014). Normal neonatal human fibroblasts (PCS-201-010™, ATCC, Manassas, VA) and normal adult human dermal fibroblasts (PCS-201-012, ATCC, Manassas, VA) were also obtained from the same vendor and cultured as per the manufacture's protocol. Cell lines were frozen in liquid vapor nitrogen at −130 °C until use. Upon thawing, cells were maintained in 5% CO_2 incubator in sterile essential media at 37 °C. Cells were then stimulated with varying concentration (0–1000 ng/mL) of TNF or IL-17 or both (Life Technologies, Grand Island, NY) for 48–72 h. Specific siRNA (Santa Cruz Biotech, Dallas, TX) mediated gene knockdown of STAT-3 (sc-29493) and cJun (sc-29223). The knockdown efficiency was measured by qPCR. All experiments were performed in triplicates.

2.2. Total protein extraction and Western blot analysis

Total proteins were extracted from cells with lysis buffer (50 mM HEPES [pH 7.6], 150 mM NaCl, 1% Triton X-100, 30 mM $Na_4P_2O_7$, 10% glycerol, 1 mM benzamidine, 1 mM DTT, 10 μg of leupeptin/mL, 1 mM phenylmethylsulfonyl fluoride 50 mM NaF, 1 mM sodium orthovandate, 10 mM sodium pyrophosphate decahydrate, 10 mM β-glycerophosphate (Sigma Aldrich, MO) (Tiriveedhi et al., 2012a). After cell lysis, the supernatant was collected and run at 15,000 × *g* for 15 min at 4 °C.

2.3. Nuclear protein extraction

Nuclear proteins were extracted from HepG2 cells with initial resuspension in Buffer 1 with protease and phosphatase inhibitors (100 mM HEPES, 5 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 10 μg/mL of aprotinin A, 10 μg/mL of leupeptin, 1 mM PMSF, 50 mM NaF, 1 mM sodium orthovandate, 10 mM sodium pyrophosphate decahydrate, 10 mM β-glycerophosphate) and later resuspended in equivolume mix of Buffer 1 and Buffer 2 (12.25 mL of Buffer 1 + 250 μL NP-40) to extract the cytosolic proteins from the supernatant. The resultant pellet of cell lysates were centrifuged at 12,000 × *g* for 1 min at 4 °C, and the remaining nuclear material were solubilized in Buffer 3 (250 mM HEPES, 350 mM NaCl, 10% sucrose, 1 mM EDTA, 1 mM DTT, 10 μg/mL of aprotinin A, 10 μg mL of leupeptin, 1 mM PMSF) (Tiriveedhi et al., 2012a).

Protein concentration was determined with a Bradford assay kit from Bio-Rad (Philadelphia, PA). Total proteins were separated on a 4–12% sodium dodecyl sulfate-polyacrylamide gradient gel and electrophoretically transferred onto a nitrocellulose membrane. The membranes were blocked overnight at 4 °C in Tris-buffered saline with 0.05% Tween 20 (5% nonfat milk in 10 mM Tris–HCl-100 mM NaCl-0. 1% Tween 20, pH 7.4). The membranes were incubated first with Abs specific for total and phosphorylated forms at room temperature with primary Abs diluted 1 in 1000 in blocking buffer for 2 h, and then with a horseradish peroxide-conjugated secondary IgG mAb diluted 1 in 5000 for 1 h. All primary and secondary Abs were obtained from Santa Cruz Biotech (Dallas, TX). The following specific primary antibodies to periostin (sc-67233), STAT-3 (sc-482), c-Jun (sc-1694), and Actin (sc-10731) were utilized. Phosphorylated forms were probed with phospho-specific primary antibodies: Ser-727-p-STAT-3 (sc-21876)Thr-183/Tyr185/c-Jun (sc-293136), respectively. The membrane was developed using the chemiluminescence kit (Millipore) and analyzed on using Bio-Rad Universal Hood II (Hercules, CA). Morphometric analysis was done using the software provided by the company.

2.4. mRNA expression analysis

Expression profiles of intracellular signaling genes in the HepG2 cells were analyzed using the FAM-labeled RT-PCR primers for Periostin (Hs01566748_m1), c-Jun (Hs99999141_s1), Stat-3 (Hs01051722_s1), GADPH (Hs402869), Actin (Hs4333762T), and Type I collagen (Hs01103892_g1) obtained from Applied Biosystems/Life Technologies (Grand Island, NY) as per the manufacturer's recommendation. Briefly, total RNA was extracted from 10^6 cells using TRIzol reagent (Sigma-Aldrich, St Louis, MO) (Tiriveedhi et al., 2012b). RNA samples were quantified by absorbance at 260 nm. The RNA was reverse-transcribed and RT-PCR (real time PCR) was performed in a final reaction volume of 50 μL using iCycler 480 Probes Master (Roche Diagnostics, Indianapolis, IN). Each sample was analyzed in triplicate. Cycling